

B. G. de los Reyes · S. J. Myers · J. M. McGrath

## Differential induction of glyoxylate cycle enzymes by stress as a marker for seedling vigor in sugar beet (*Beta vulgaris*)

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**Abstract** Significant differences in seedling vigor exist among sugar beet (*Beta vulgaris*) hybrids; however, traditional approaches to breeding enhanced vigor have not proven effective. Seedling vigor is a complex character, but presumably includes efficient mobilization of seed storage reserves during germination and efficient seedling growth in diverse environments. The involvement of lipid metabolism during germination of sugar beet under stress conditions was suggested by the isolation at high frequency of Expressed Sequence Tags (ESTs) with similarity to isocitrate lyase (EC 4.1.3.1). High-level expression of this glyoxylate cycle enzyme during germination and seedling emergence was also suggested by nucleotide sequencing of cDNA libraries obtained from a well emerging sugar beet hybrid during germination under stress. Genes involved in carbohydrate and lipid catabolism were differentially expressed in a strongly emerging hybrid, relative to a weakly emerging hybrid, during stress germination. Stress markedly reduced the levels of  $\alpha$ -amylase transcripts in the weakly emerging hybrid. In contrast, the strongly emerging hybrid exhibited only a moderate reduction in  $\alpha$ -amylase transcript levels under the same conditions, and showed large increases in the expression of genes involved in lipid metabolism, suggesting compensation by lipid for carbohydrate metabolism in the better emerging hybrid. Differential activity of the glyoxylate cycle thus appears to be a physiological marker that

distinguishes between high- and low-vigor sugar beet cultivars. This finding suggests, for the first time, a biochemical target for selection for enhanced germination and improved emergence in sugar beet.

**Keywords** Anaplerotic pathway · Germination · Isocitrate lyase · Seedling vigor · Stress tolerance

### Introduction

Low seedling emergence due to poor vigor is a frequent problem in direct seeded crops such as sugar beet. Sugar beet hybrids differ in seedling vigor (McGrath et al. 2000), and the ability to germinate and emerge vigorously under a wide range of conditions has a complex physiological basis (Bewley and Black 1994; Holdsworth et al. 1999; Thomas 1993). Indeed seedling vigor in sugar beet is so profoundly influenced by environment that no heritability estimates are available. Our objectives have been to examine the germination of sugar beet in order to identify potential physiological and developmental targets for traditional and marker-assisted breeding for enhanced germination potential. As one approach, Expressed Sequence Tags (ESTs) were generated from cDNA libraries constructed from RNA isolated from a high-vigor sugar beet hybrid, with the intention of identifying potential mediators of enhanced germination based on gene expression profiles. We were particularly interested to see if multiple gene transcripts for components of a single pathway might be present among the ESTs.

Stress imposed by germinating sugar beet in solution mimics the relative emergence of different varieties and seedlots in the field. Differences in the efficiency of germination in solution between well emerging and poorly emerging cultivars are, however, reduced, and sometimes eliminated, when the germination medium is supplemented with  $H_2O_2$  (McGrath et al. 2000). Cultivar differences in seedling vigor may be due in part to induced expression of a germin-like protein (a putative  $H_2O_2$ -producing oxalate oxidase), which, in high-vigor

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B. G. de los Reyes · S. J. Myers · J. M. McGrath (✉)  
USDA-ARS, Sugar Beet and Bean Research Unit,  
Michigan State University, 494 Plant and Soil Sciences Building,  
East Lansing, MI 48824-1325, USA  
E-mail: mitchmcg@msu.edu  
Tel.: +1-517-432-2355  
Fax: +1-517-337-6782

*Present address:* B. G. de los Reyes  
Department of Crop, Soil, and Environmental Sciences,  
University of Arkansas, 115 Plant Science Building,  
Fayetteville, AR 72701 USA

germplasm, could be physiologically equivalent to externally supplied  $H_2O_2$  (de los Reyes and McGrath 2003). Other mechanisms are also likely to influence seedling vigor. Evidence reported here suggests that differential activity of carbohydrate and lipid catabolic pathways in seeds germinating under stress is of physiological significance, and has potential applications in attempts to enhance seedling vigor in sugar beet, and perhaps other small-seeded species.

Traits with low heritability, such as yield in most crops, are among the most difficult to dissect genetically. The approach described here may help to augment progress in identifying genes associated with low heritability traits, at least where clear varietal differences exist in controlled environments. Genes identified in this manner would then become candidates that require additional confirmation by examining gene expression differences and ultimately by demonstrated progress in breeding for the trait of interest. In the present report, candidates have been identified and demonstrated between sugar beet germplasm that shows variation in seedling vigor, and these provide promising targets for selection for seedling emergence, which is otherwise a low-heritability trait.

## Materials and Methods

### Seed germination

Good-quality seedlots (average germination >92%) of the commercial hybrids USH20 (strongly emerging; Coe and Hogaboam 1971) and ACH185 (weakly emerging) (American Crystal, Moorhead, Minn.) were used in this study. The same seedlots were used throughout these experiments. It is important to note that both ACH185 and USH20 were once dominant commercial hybrids grown in Michigan, and that emergence and stand establishment were not the primary reasons for their being supplanted by newer hybrids (the reasons were substantially related to disease pressure). These two hybrids were chosen because they differ markedly in their germination characteristics under stress, both in the field and in solution (McGrath et al. 2000; de los Reyes and McGrath 2003). Control (unstressed) germination tests were performed by allowing 25 seeds (four replications per seedlot) to germinate on moistened pleated filter paper (TeKrony and Hardin 1966). Germination tests in solution (four replications of 25 seeds each, in 125-ml flasks) were performed at 23°C in 15-ml volumes, using 88 mM  $H_2O_2$ , highly purified water (18 MOhm), or 150 mM NaCl (McGrath et al. 2000). Polished, untreated seeds were immersed without pretreatment at time zero, and continuously shaken at 100 rpm on a rotary shaker, with changes of solution daily. Germination (radicle length >2 mm) was followed daily for 8 days. Germination was considered as stressful when the mean percentage of treated seeds that germinated was statistically significantly less than mean control germination percentages; this occurred only in pure water and in NaCl solution. Seedlings used for biochemical extractions were subjected to the same conditions; however, volumes were scaled up (~500 seedlings per 200 ml solution in 1-l flasks).

### cDNA library construction and hybridization with subtracted probes

Poly(A)<sup>+</sup> RNA was purified using the PolyAtract mRNA isolation kit (Promega) from total RNA isolated with guanidine

hydrochloride from ~1500 4-day-old USH20 seedlings (Logeman et al. 1996). Equal amounts of poly(A)<sup>+</sup> RNA (2 µg) from salt and  $H_2O_2$  germination regimes were pooled to construct a directional cDNA library in Lambda Uni-Zap (Stratagene). From the primary library,  $1 \times 10^5$  pfu were excised as pBluescript phagemids, and plated at a density of 4000 cfu per 150 mm plate. Colony lifts were prepared with Hybond N<sup>+</sup> membrane (Amersham Biosciences).

Subtracted cDNA probes were prepared by biotin-streptavidin subtraction (Sive and St. John 1988) using the Subtractor kit (Invitrogen). Two types of induced poly(A)<sup>+</sup> RNA samples (germinated in 150 mM NaCl, and germinated in 88 mM  $H_2O_2$ ) were used as testers for subtraction against control poly(A)<sup>+</sup> RNA driver from seedlings germinated for 4 days on moist filter paper. Briefly, 1 µg of each tester poly(A)<sup>+</sup> RNA pool was reverse transcribed to first-strand cDNA with oligoT primer and MMLV reverse transcriptase. The driver probe was prepared by labeling a ten-fold excess of control poly(A)<sup>+</sup> RNA with photobiotin. Subtraction was performed by hybridizing the tester and driver probes in 2× hybridization buffer at 65°C for 48 h. After hybridization, the cDNA species that were common to the tester and driver pools (cDNA/mRNA-biotin hybrids) were removed by streptavidin coupling and phenol-chloroform extraction. The subtracted cDNA pool was labeled with [<sup>32</sup>P]dCTP by random priming (Ausubel et al. 1988). Colony lifts were hybridized with subtracted cDNA probes at 65°C for 48 h in Denhardt's solution supplemented with 5×SSC and 0.5% SDS, washed at 65°C with 2× SSC/0.1% SDS and 0.25×SSC/0.1% SDS, and then autoradiographed at -80°C for 4–6 days. Colonies that hybridized with subtracted probes were subjected to nucleotide sequencing.

### cDNA sequencing and EST analysis

The subtracted library was sequenced with the aid of an ABI 3700 (Applied Biosystems). Sequence tags were annotated by BLASTN and BLASTX alignments with the non-redundant (nr) and EST (dBEST) sequence datasets (Altschul et al. 1997). ESTs were classified by biochemical function based on the Kyoto Encyclopedia of Genes and Genomes graphical pathway maps (<http://www.genome.ad.jp/kegg/>; Kanehisa and Goto 2000), using a threshold e-value of  $10^{-10}$  or better for preliminary assignment of a putative function.

### Northern hybridization analysis

Total RNA was isolated from ~500 control or solution-germinated seedlings 3–6 days after immersion, and 10-µg samples were fractionated on a 1.2% formaldehyde-agarose gel and blotted onto Hybond N membrane. RNA filters were hybridized overnight at 42°C in NorthernMax buffer (Ambion) with <sup>32</sup>P-labeled cDNA probes for α-amylase (BF011027), acetyl-CoA acyltransferase (BI096013), isocitrate lyase (BF011213), malate synthase (BI073189), a seedling vigor associated germin-like protein (AF10017), and EF-1α (BI643084, used to monitor gel loading). Ethidium bromide-stained ribosomal RNA bands were also used as gel loading controls. Filters were washed in 2×SSC/0.1% SDS and 0.5×SSC/0.1% SDS, and autoradiographed.

### Isocitrate lyase activity assay

Soluble protein extracts from control and solution-germinated seedlings were prepared 2–8 days after immersion. The seed coats were removed and whole seedlings were homogenized (1:1 vol/vol) in TRA buffer (80 mM triethanolamine, 10 mM MgCl<sub>2</sub>, 2 mM EDTA, 2 mM dithiothreitol, pH 7.5) with 1% Tween 80 (Pinzauti et al. 1982). The homogenate was filtered through cheesecloth and centrifuged at 20,000× g for 20 min. Total protein content of the supernatant was quantified with Bradford reagent (Biorad). Isocitrate lyase activity was determined by the lactate

dehydrogenase-coupled continuous assay (Giachetti et al. 1983), performed at 37°C in 40 mM HEPES (pH 7.0), 6 mM MgCl<sub>2</sub>, 4 mM *threo*-D,L-isocitrate, 0.28 mM NADH and 45 U of LDH (glyoxylate substrate). NADH oxidation was monitored at 340 nm. The results represent the means of two measurements from independent samples, and are expressed in units of enzyme activity per milligram protein, based on a standard curve.

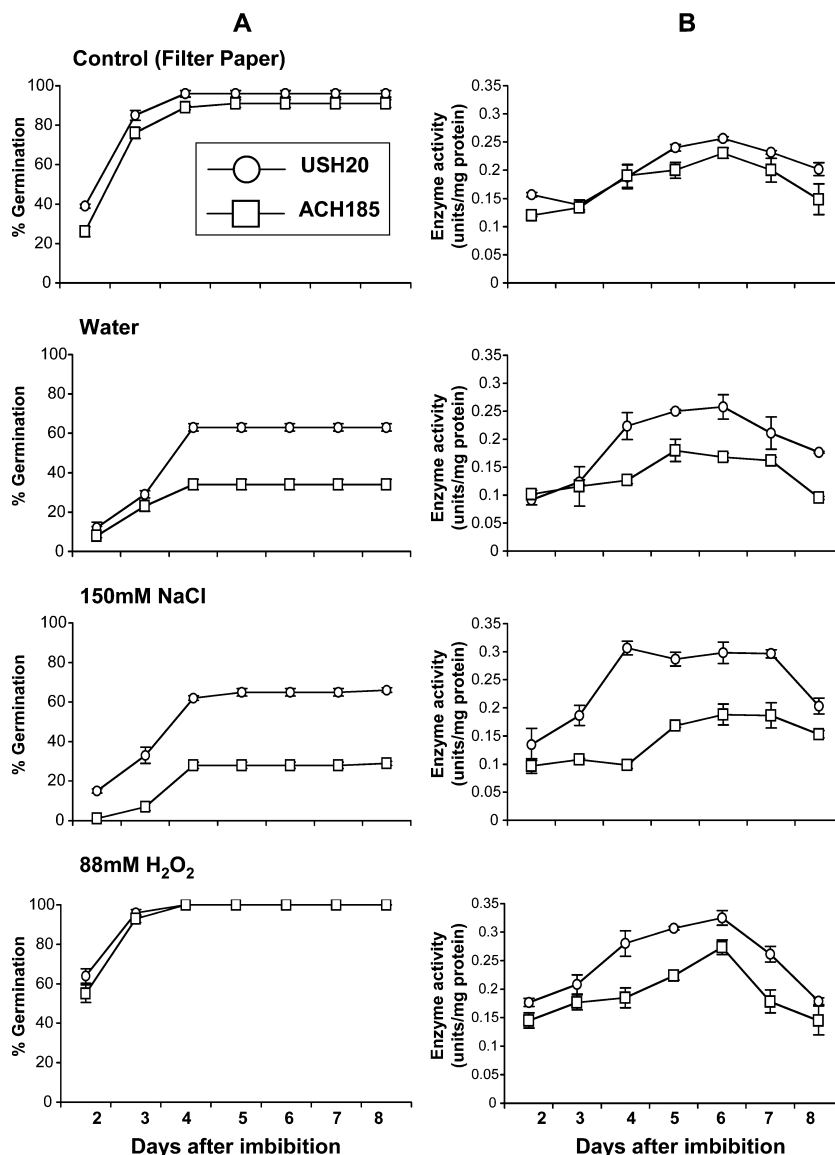
## Results

### Characterization of EST libraries constructed with RNA derived from sugar beet seedlings germinated under stress

ESTs (1853 in total) were obtained from 4-day-old, solution-germinated seedlings of the well emerging sugar beet variety USH20 to obtain an initial assessment of genes likely to be involved in enhanced germination under stress. Two conditions of solution germination

were used for cDNA construction, one (NaCl) that reduced germination efficiency in solution and another (H<sub>2</sub>O<sub>2</sub>) that increased germination percentages in solution, relative to emergence in water (Fig. 1A). Briefly, 823 ESTs were obtained from a cDNA library constructed from equal mixtures of mRNA isolated from seedlings germinated in salt or H<sub>2</sub>O<sub>2</sub> solution (i.e. unsubtracted library), 645 ESTs were obtained from NaCl-germinated seedlings after subtraction with mRNA from control germinated seedling tissue (i.e. subtracted NaCl), and 385 ESTs were obtained from H<sub>2</sub>O<sub>2</sub>-germinated seedlings subtracted with the same control mRNA (i.e. subtracted H<sub>2</sub>O<sub>2</sub>). All ESTs have been deposited in dbEST (<http://www.ncbi.nlm.nih.gov/dbEST/>). Most ESTs were represented as one or a few members of a functional class (1016 singletons/1853 total = 54.8%). Subtraction was slightly better in recovering unique ESTs than was sequencing the unsubtracted library, as 59.7% of subtracted NaCl, 55.8%

**Fig. 1A, B** Time course of germination (**A**) and isocitrate lyase activity (**B**) in the cultivars USH20 and ACH185 under different environmental conditions. **A** Data for each time point are averages of four independent germination experiments. **B** Isocitrate lyase activity at different time points during germination and early seedling growth of USH20 and ACH185. Data for each time point are averages of two independent measurements. The vertical bars represent the standard error of the mean



of subtracted  $H_2O_2$ , and 50.5% of unsubtracted ESTs were unique among the total dataset. The remaining ESTs fell into one of 427 clusters, with the largest cluster of 33 ESTs showing similarity to translation elongation factor EF-1 $\alpha$  (Berberich et al. 1995). Our intention in this report was not to describe these ESTs in detail, but to highlight a previously unconsidered aspect of seedling vigor in sugar beet—that of utilization of stored energy reserves—which only became apparent upon inspection of this collection of ESTs.

Grouping according to biochemical function showed that 7.2% of ESTs represented genes known to be involved in carbohydrate or lipid catabolic pathways. As expected for carbohydrate utilization, transcripts encoding starch and polysaccharide hydrolases and debranching enzymes were numerous (i.e. present at a frequency of 1.5% of all ESTs). Transcripts for  $\alpha$ -amylase were the most abundant EST for this functional category (0.3% of ESTs). However, not all genes involved in major sugar catabolism pathways (e.g. glycolysis, tricarboxylic acid cycle) were present, but active pathways were indicated by the presence of ESTs encoding some of their diagnostic enzymes (3.8% of ESTs).

The importance of lipid metabolism during solution germination was indicated by the relatively high frequency of transcripts for lipases and fatty acid hydrolases (0.6% of ESTs). Activity of the fatty acid  $\beta$ -oxidation spiral was indicated by an EST for acetyl-CoA acyl transferase. Glyoxylate cycle pathway activity was indicated by a relatively high percentage of transcripts (1.2%) for the glyoxysomal enzymes isocitrate lyase (ICL) and malate synthase (MS), as well as for aconitase. Isocitrate lyase is the key enzyme of the glyoxylate cycle, linking fatty acid oxidation and sugar metabolism in germinating oil seeds (Goodwin and Mercer 1983).

#### Gene expression profiles during seedling germination

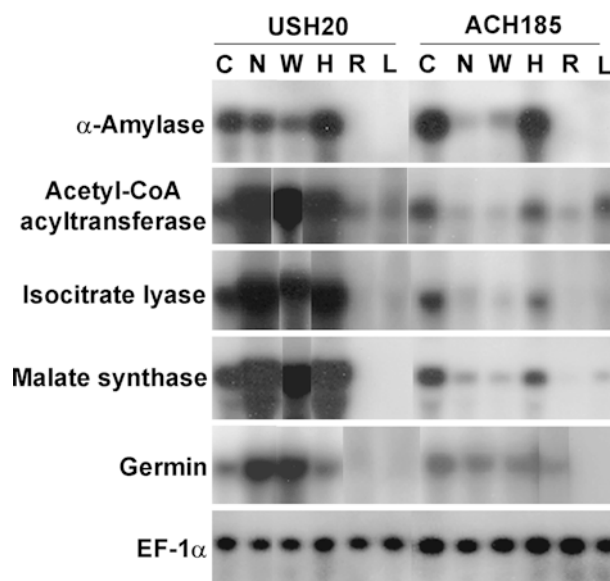
The sugar beet hybrids USH20 and ACH185 represent extremes of field emergence potential (e.g. seedling vigor) among the commercial hybrids tested (McGrath et al. 2000). These two hybrids showed markedly different percentages of germination in water and in salt solution, but not on filter paper or in hydrogen peroxide solution (Fig. 1A). Germination was essentially complete by 4 days after immersion. Phenotypically, salt and water treatments were thus considered stress conditions, and filter paper and hydrogen peroxide were non-stress conditions.

ICL activity was followed over a period of 8 days post-immersion (Fig. 1B). Isocitrate lyase activity was maximal at 6 days post-immersion in both non-stress conditions, i.e. on filter paper and in  $H_2O_2$  solution. Under stress conditions (germination in water or salt), ICL activity in the less vigorous hybrid ACH185 was always less than that in the more vigorous USH20, with the exception of a non-significant difference in water at

2 days post-immersion (Fig. 1B). However, maximal ICL activity in ACH185 was reached 1 day earlier than in USH20 in seeds germinating in water, but 2 days later in the salt-stressed samples. The significance of these differences in the timing of maximal ICL activity between the strongly and weakly emerging hybrids, if any, is not yet clear.

Analysis of gene expression by RNA gel-blot hybridization showed variation in the abundance of EST transcripts in whole organs (i.e. seedlings, leaves, roots), 4-day-old germinating seedlings (i.e., in water, salt or  $H_2O_2$ , or on filter paper), as well as between USH20 and ACH185 (Fig. 2). Five gene products were monitored:  $\alpha$ -amylase (as a marker for carbohydrate catabolism in germinating seeds); acetyl-CoA acyltransferase (a  $\beta$ -oxidation enzyme that links lipid metabolism with the glyoxylate cycle); ICL and MS as markers for the glyoxylate pathway; and a germin-like protein as a sugar beet cultivar-specific marker for stress response and seedling vigor (de los Reyes and McGrath 2003). Additional transcripts coding for key enzymes of glycolysis (fructose biphosphate aldolase, phosphoglucumutase, triose phosphate isomerase, and glyceraldehyde 3-phosphate dehydrogenase) were also monitored, but RNA gel blots showed that transcripts coding for these enzymes were highly abundant in both hybrids, with a slight induction in solution, and thus did not exhibit clear cultivar specificity at this level of analysis (data not shown).

In roots and leaves, levels of ICL and MS transcripts were relatively low or undetectable in both hybrids, and



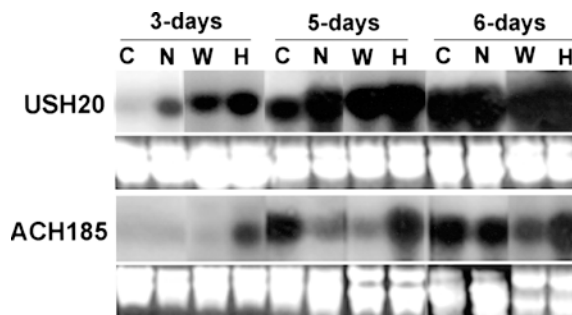
**Fig. 2** Transcript abundances in USH20 (good emerger) and ACH185 (poor emerger) for the key enzymes of the glyoxylate cycle and for some enzymes involved in carbohydrate and lipid metabolism in 4-day-old seedlings. (C = control = filter paper germination; N = salt stressed 150 mM NaCl; W = complete submergence in water; H = 88 mM  $H_2O_2$ ). Also shown are the transcript abundances in roots (R) and leaves (L) of mature plants (60 days old). Hybridization with a probe for elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) was used as loading control

similar observations were made for transcripts of  $\alpha$ -amylase, acetyl-CoA acyl transferase, and germin-like protein genes (Fig. 2), which is consistent with their more specific role in germination.

Large differences in transcript abundance were observed between germination treatments, as well as between high- and low-vigor hybrids. Levels of  $\alpha$ -amylase transcripts were high in both hybrids under non-stress conditions (i.e. on filter paper and in  $H_2O_2$ ). Seeds subjected to non-stress conditions also showed a relatively high abundance of transcripts for each of the other enzymes, although these levels appeared higher in the well emerging USH20, and this difference was more pronounced when germination occurred in  $H_2O_2$ . With the exception of USH20 germinating in  $H_2O_2$ , transcript levels were roughly equivalent between the varieties USH20 and ACH185 under unstressed germination conditions, as judged by visual comparison of signal intensities.

Under stress conditions (water and salt germination), a marked difference in transcript abundance was evident between USH20 and ACH185, which was not seen in the control treatments (Fig. 2). Expression of  $\alpha$ -amylase was reduced in USH20, but not to the extent observed for ACH185. Interestingly, a greater reduction in the levels of  $\alpha$ -amylase transcripts (relative to the filter paper condition) was observed in water-treated than in salt-treated USH20, and this appeared reversed in ACH185. Each of the other four gene products assayed was highly abundant in stressed (water and NaCl) USH20 relative to the filter paper control, and often substantially reduced in ACH185 relative to its filter paper control. The magnitude of this difference as judged visually by comparison of signal intensities suggested that the enzymes encoded by these genes might be important for the expression of seedling vigor in sugar beet. Furthermore, increased signal intensity relative to the filter paper sample in USH20 suggested an induction of gene expression for lipid metabolizing enzymes, while the reduction in signal intensity relative to the filter paper control in ACH185 suggested a repression of genes for these same enzymes.

The temporal pattern of ICL expression was followed over 6 days, to determine whether the timing of lipid reserve mobilization was related to differential germination of USH20 and ACH185 in solution relative to filter paper (Fig. 3). In seedlings germinated on filter paper, barely detectable levels of ICL transcripts were seen in either hybrid at 3 days post-imbibition, and their levels increased and appeared roughly equivalent at 5 and 6 days post-immersion in both hybrids. ICL transcript levels followed the same trend in  $H_2O_2$  germinated seedlings, except that transcripts were already abundant by the 3-day timepoint. In seedlings germinated under stress (in water or salt), USH20 showed ICL transcripts in abundance at 3 days post-imbibition, while they were barely detectable in ACH185. At 5 and 6 days post-imbibition, ICL signal intensities were higher in USH20 than ACH185, with the exception perhaps of ACH185



**Fig. 3** Comparison of the temporal expression patterns of isocitrate lyase mRNA (upper panels) between USH20 and ACH185. (C = control = filter paper germination; N = salt stressed, 150 mM NaCl; W = complete submergence in water; H = 88 mM  $H_2O_2$ ). Ethidium bromide-stained rRNA bands are shown (lower panels) as a loading control

germinated in salt at 6 days relative to USH20 at the same time. Interestingly, ICL expression in ACH185 appeared increased in salt relative to water, especially at 6 days; perhaps this observation is relevant to the outdated agronomic practice of using NaCl as a sugar beet fertilizer (e.g. Allen et al. 1978).

## Discussion

Seedling vigor involves the a priori coordinated regulation of numerous genes with essential physiological and developmental roles. Efficient use of energy reserves to enable continuous heterotrophic growth is fundamental for rapid establishment (Hwang et al. 1999). Here we report on the relative abundances of ESTs derived from genes for the key glyoxylate cycle enzymes isocitrate lyase and malate synthase in seedlings germinated in NaCl and  $H_2O_2$ -solutions. Our data raise the question of the importance of lipids as an energy source during germination and seedling emergence under sub-optimal conditions. In oil seeds, among which sugar beet is not traditionally classified, the glyoxylate cycle links lipid and carbohydrate metabolism during germination and early seedling growth. This link occurs via succinate produced from the condensation of two acetyl-CoA molecules in the glyoxysome (Eastmond and Graham 2001; Kornberg and Krebs 1957), providing carbon intermediates from lipid metabolism for the biosynthesis of transportable sucrose, and replenishing the TCA cycle under conditions in which most intermediates are being withdrawn for biosynthetic processes (i.e. an anaplerotic function).

Germination and emergence of sugar beet under sub-optimal conditions appears to be associated with reduced  $\alpha$ -amylase expression, and varietal differences between the extreme high- and low-vigor hybrids used here may be explained in part by an increase in fatty acid oxidation and glyoxylate cycle activities in the strongly emerging hybrid as a mechanism to compensate for reduced availability of carbohydrate-derived carbon

sources. Starvation for sugar is a potent positive regulator of the glyoxylate cycle (Graham et al. 1994a). Expression of ICL and MS is regulated at the transcriptional level, and responds to various metabolic signals (Zhang et al. 1996; Graham et al. 1994b; Allen et al. 1988). The EST collection presented in this study is far from being representative of the total transcriptome in germinating sugar beet, and the precise regulatory mechanisms that link stress response and energy metabolism in germinating sugar beet on a global scale may become available through the application of more comprehensive genomic and proteomic approaches (e.g. Gallardo et al. 2001).

Whether lipids provide a sufficient carbon resource for sugar beet germination is a question for further investigation. Chemical analyses of sugar beet seed, particularly lipids, are scarce. Ware (1898) refers to a number of chemical constituents of beet seed, and indicated a starch content of 39.6%, primarily located in the perisperm (a maternally derived endosperm-like tissue), a protein content of 27.6% primarily located in the embryo, and 20.5% lipid content distributed throughout the perisperm and embryo. In contrast, Elamrani et al. (1992) indicated that there is virtually no lipid in the perisperm but ~15% lipid content in the embryo, and showed that lipids are likely to be the initial substrate for respiration during germination of sugar beet, with carbohydrates assuming greater importance after radicle protrusion from the seed ball. Similarly, Lawrence et al. (1990) showed differences in organ-specific starch, protein, and sugar contents in excised seeds and seedlings, and suggested a specialization of the inner cotyledon (in closest proximity to the perisperm) for carbohydrate uptake. Differences between varieties in lipid and carbohydrate content may account for differences in emergence potential. However, the evidence presented here suggests that other layers of control, at the level of gene expression and perhaps in enzyme activities and signal transduction (de los Reyes and McGrath 2003), also need to be considered for the enhancement of field emergence potential in sugar beet.

The initial supposition that genomic approaches can be directly applied to practical breeding objectives appears to be reasonable, as these strategies suggest potential targets for biochemical selection for enhanced emergence in this case. Selection ought to be practiced most effectively if the precise mechanisms of trait expression are known for the particular environments (or in validated proxy environments) where the trait is economically vulnerable. Practical breeding programs should continue to develop and deploy genomic tools for their specific crops, as is occurring in many instances, and perhaps assume greater leadership in the application of these tools. For the practical goal of enhancing sugar beet emergence, a problem for growers worldwide, gene expression trends exhibited by glyoxylate cycle enzymes provide clues to a perhaps rather intricate mechanism. Cultivar-dependence of glyoxylate cycle activity suggests

that stress-induced expression of ICL is an indicator of seedling emergence potential and enhanced vigor in sugar beet.

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